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P16 and the *melanocortin 1 receptor* genes are two important melanoma predisposition genes. We hypothesize that co-inheritance of certain germline mutations in *p16* and *MC1R* RHC alleles synergistically sensitizes melanocytes to the damaging effects of UV , and thus increases the chance for malignant transformation to melanoma, by inhibiting autophagy and senescence, and increasing oxidative stress by activating NFκB. Our present results using melanocyte cultures from donors heterozygous for a *p16* mutation, with or without co-expression of a *MC1R* non-functional allele, did not differ markedly from melanocytes wild type for *p16* and *MC1R,* or heterozygous for a non-functional *MC1R* variant in their proliferation capacity, repair of DNA photoproducts, growth arrest, and generation of hydrogen peroxide following a single acute UV exposure. Our results suggest that expression of one functional *p16* and *MC1R* allele is sufficient for maintaining melanocyte homeostasis. We will investigate the response of mutant melanocytes to chronic UV exposure, which might overwhelm their compensatory mechanisms and drive their malignant transformation. These studies are particularly relevant to military personnel with family history of melanoma or a prior melanoma, who are deployed in areas with excessive sun exposure, and should improve melanoma risk assessment based on *p16* and *MC1R* genotypes.

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1.Introduction:

P16 and *MC1R* are *bona fide* melanoma susceptibility genes. This project aims at investigating the hypothesis that co-inheritance of certain germline mutations in *p16* and *melanocortin 1 receptor* (*MC1R*) red hair color (RHC), loss-of-function alleles synergistically sensitizes melanocytes to the damaging effects of UV , and thus increases the chance for malignant transformation to melanoma, by inhibiting autophagy, senescence, and increasing oxidative stress by preventing inhibition of NFκB. Germline mutations in *p16* are the most prevalent in familial melanoma, and somatic mutations in *p16* or epigenetic modification of *p16* expression are also present in sporadic melanoma. At least 48 different *p16* germline mutations have been identified. P16 is a cyclin-dependent kinase (CDK) 4/6 inhibitor, which blocks cells in G1 phase of the cell cycle. It is also involved in regulating nucleotide excision repair (NER) and reducing cellular oxidative stress and repair of oxidative DNA damage. The *MC1R* codes for a G_s protein-coupled receptor that is expressed on the cell surface of melanocytes. Activation of this receptor by its agonist α-melanocortin (α-MSH) stimulates the synthesis of the photoprotective brown/black eumelanin. More than 100 allelic variants of the *MC1R* have been identified in different human populations. Some variants of the *MC1R*, R151C, R160W, and D294H, are strongly associated with red hair color, fair skin, and poor tanning ability. Expression of any two of these alleles results in loss of function of the MC1R in melanocytes. We discovered that activation of the MC1R reduces UV-induced DNA damage by inhibiting the generation of reactive oxygen species (ROS) and enhancing repair of DNA photoproducts and oxidative DNA damage in human melanocytes independently of pigmentation. These results provide experimental evidence for the association of loss-of-function *MC1R* alleles with increased melanoma susceptibility, and reveal common pathways, such as NER and antioxidant pathways, that are affected by both p16 and MC1R. To investigate our hypothesis, we have been testing a panel of primary cultures of human melanocytes that were established prior to obtaining funding from CDMRP. We had established primary cultures of melanocytes, keratinocytes and fibroblasts form skin biopsies of patients from melanoma-prone families, who are heterozygous for one of 3 melanoma-associated *p16* mutations, and either wild type *MC1R* or heterozygous for one of the loss-of-function *MC1R* variants (Table 1). The biopsies were obtained by my collaborators Sancy Leachman, M.D./Ph.D., and Pamela Cassidy, Ph.D., when they were faculty members in the Department of Dermatology at University of Utah, and Dr. Leachman was the Director of the Melanoma Clinic at the Huntsman Cancer Institute, with approved IRB protocol and patients' consent. The IRB office at the University of Cincinnati deemed the study as "No Human Subjects", since the biopsies were totally de-identified to me. As controls, we have been using melanocyte cultures expressing wild type *p16*, together with wild type *MC1R* or one loss-of-function *MC1R* variant that are already available in my laboratory from previous projects. Co-inheritance of both *p16* and *MC1R* mutations results in early onset of melanoma and increases the penetrance of the mutant *p16*. However, the molecular mechanism(s) by which mutations in these two genes interact to drive the malignant transformation of melanocytes remains unknown. To date, there are no studies on the dual action of *p16* and *MC1R* mutations on the capacity of melanocytes to maintain genomic stability and overcome the damaging effects of UV. Our proposed studies will fill this gap in knowledge.

2.Key words:

P16
MC1R
Human melanocytes
Ultraviolet radiation
Melanoma
Cell cycle
DNA repair
Autophagy
Senescence
Oxidative stress

3. Accomplishments:

Major goals of the project:

Specific Aim I: Investigate activation of autophagy by UV in cultured human melanocytes and how this is affected by different *p16* and *MC1R* genotypes

Specific Aim II: Determine how certain mutations in *p16* and *MC1R* variants inhibit senescence in cultured human melanocytes and autologous 3-D skin substitutes

Specific Aim III. Elucidate how different *p16* and *MC1R* mutations increase oxidative stress in melanocytes by activating NFκB through preventing its inhibition

What was accomplished under these goals:

The results described below were based one experiments utilizing pre-existing melanocyte cultures listed in Table 1.

Results relevant to Specific Aim I.

We have tested whether or not melanocyte cultures expressing a mutant *p16* allele, and either wild type or loss-of- function *MC1R* variant efficiently repair UV-induced DNA photoproducts. Upon testing 2 cultures, 17494 and 17609, we found that similar to their counterparts with wild type *p16* and functional MC1R, these cultures effectively repaired cyclobutane pyrimidine dimers (CPDs), the major form of DNA photoproducts, evidenced by reduction in CPDs 48 after exposure to 90 mJ/cm^2 UV (Fig. 1). Additionally, these cultures had a comparable percent of apoptotic cells to their counterpart with wild type *p16* and functional MC1R. We will correlate the DNA repair capacity of melanocytes with their ability to undergo autophagy. We also tested the ability of 17609 and 85627, which are heterozygous for a loss-of-function *MC1R* variant, to respond to α-MSH with enhanced CPD repair (Fig. 2). Our results indicate that treatment with α-MSH enhances CPD repair in these melanocytes, as we have previously shown in melanocytes with the same *MC1R* genotypes but wild type for *p16.*

Established cultures	Age	Gender	*p16* Genotype	*MC1R* Genotype
17490	28	M	V126D/+	+/+
17494	20	F	V126D/+	+/+
10766	42	M	V126D/+	R160W/R151C
85627	35	F	V126D/+	R160W/+
17489	31	F	V126D/+	D294H/+
17491	26	F	V126D/+	D294H/+
17609	18	M	V126D/+	D294H/+
19972	21	M	5'UTR-34G>T/+	R160W/+
19973	18	M	5'UTR-34G>T/+	R160W/+
19971	24	M	5'UTR-34G>T/+	D294H/+
84891	22	M	5'UTR-34G>T/+	V60L/T314T
87195	37	M	32ins24/+	V60L/R160W
87203	32	F	32ins24/+	V60L/R160W
87204	22	M	32ins24/+	R160W/+
P16-/- 1				
17493	23	M	+/+	+/+
26310	28	F	+/+	+/+
117C	43	F	+/+	+/+
114C		F	+/+	V92M/R151C/T314T
26671	25	F	+/+	R151C/+
59854	23	F	+/+	R151C/+
116C		F	+/+	R160W/+
59842	40	M	+/+	R160W/+

Table 1: List of primary melanocyte cultures that were established prior to the funding period, and used for the data presented in this progress report.

We gave begun to compare the ability of melanocytes with different *p16* and *MC1R* genotypes to undergo autophagy in response to UV exposure. Preliminary data from 2 different cultures with wild type *p16* and *MC1R* revealed that

autophagy was induced within 24 h post irradiation with 75 or 90 mJ/cm^2 UV, as depicted by increased expression of the autophagy marker LC3II, using Western blot analysis. Representative data are presented in Fig. 3.

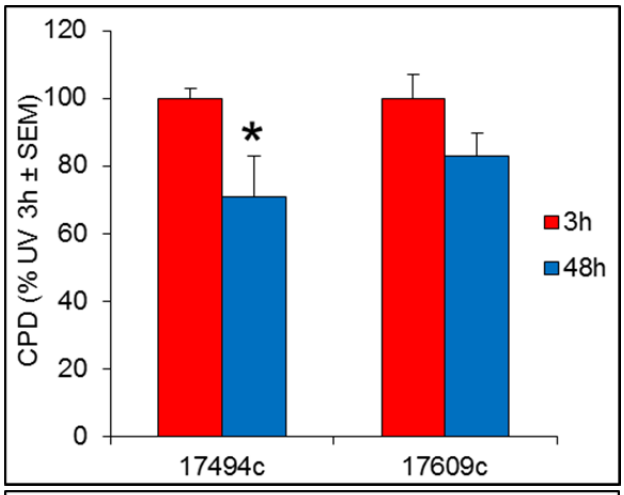

Figure 1. Repair of CPDs in melanocytes heterozygous for *p16* mutation, with or without expression of a mutant *MC1R* allele. Melanocytes were irradiated with a dose of 90 mJ/cm^2 UV, harvested and fixed 3 and 48 h thereafter, and stained with a CPD-specific antibody to determine induction and repair of CPDs, respectively. Melanocytes were analyzed by flow cytometry, with 10,000 events analyzed per sample, and 3-5 samples included in each group. Each data point represents the mean of 3-5 different samples/group ±S.E. Data is expressed as percent of the respective UV 3 h group, which is considered 100%. *=Statistically different from the respective UV 3h at p<0.01.

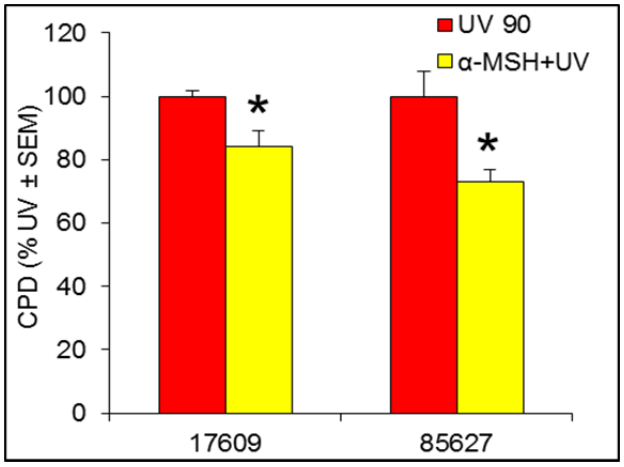

Figure 2. Enhancement of CPD repair in melanocytes co-expressing one *p16* and one *MC1R* mutant allele by α-MSH treatment. Melanocytes were pretreated with 0, or 10 nM α-MSH, the physiological MC1R agonist, for 4 days prior, and for 2 days post irradiation with 90 mJ/cm^2 UV. Melanocytes were harvested and fixed 3 days post UV, and stained and analyzed for CPDs. Each data point represents the mean of 3-5 different samples/group ±S.E., and is expressed as percent of the UV group. *=Statistically different from the respective UV group at p<0.01.

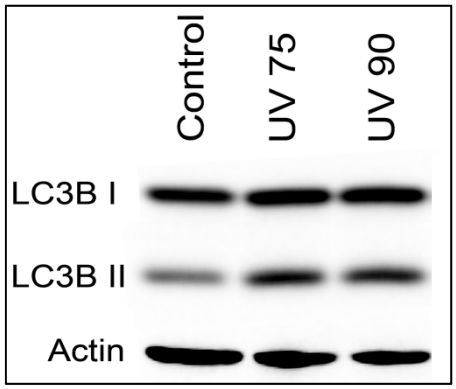

Figure 3. Induction of autophaghy in melanocytes by UV irradiation, as depicted by increased levels of LC3II protein. Western blot analysis was carried out on total cell lysates of melanocytes 24 h post irradiation with 75 or 90 mJ/cm^2 UV. Actin was used as a loading control.

Conclusion: These results suggest that expression of one mutant *p16* allele does not compromise repair of DNA photoproducts or the ability of melanocytes, specifically those heterozygous for a loss-of-function *MC1R* variant, to respond to α-MSH with enhanced repair of CPDs. Our preliminary data provide proof-of-principle that UV induces autophagy in cultured human melanocytes.

Our goal as listed in the SOW was to accomplish the experiments described for Specific Aim 1 during the first 9 months of funding. We are currently moving steadily on the experiments aimed at comparing autophagy in melanocytes with different genotypes. We had elected to proceed earlier than we had initially planned with Tasks 1 and 3 of Specific Aim II, and Task 1 of Specific Aim III that was due to start during the second year of funding.

Drs. Leachman and Cassidy moved from University of Utah to Oregon Health Sciences University (OHSU) during the summer of 2014. The delay in starting the recruitment of patients to obtain additional biopsies was due to the time it took them to establish their clinic and laboratory and to obtain IRB approval from their new institution. We had submitted IRB approvals from my own institution and OHSU to CDMRP officials. Additional documentation was required by CDMRP personnel reviewing the Human Subjects status of the award, and that has been submitted to CDMRP. We are still waiting for the final DOD approval of the use of human subjects by OHSU, which was designated by DOD officials as a second performance site.

Cell Strain	*P16* Genotype	*MC1R* Genotype	% β-gal positive cells
17490, P.4	V126D/+	+/+	2.2±0.3
17494, P.9	V126D/+	+/+	32.2±3.4
85627, P.10	V126D/+	R160W/+	13.4±2.1
105, P.9	+/+	+/+	18.5±4.9
1584, P.6 *	+/+	+/+	17.5±0.9
29244, P.10	+/+	D294H/+	75.3±11.4
49422, P.10	+/+	D294H/V92M	93.8±1.2

Table 2. Quantification of the percent of senescent melanocytes (±S.E.) in cultures with different *P16* and *MC1R* genotypes, as determined by β-galactosidase staining. The last two cultures were adult melanocytes that stopped proliferating, and hence were used as positive control.

Results relevant to Specific Aim II.

We have begun to quantify the percent of senescent melanocytes in culture, using β-galactosidase stain (Task 1). Out of 3 *p16* mutant cultures, only one showed markedly reduced percent of β-galactosidase-positive cells, the second had comparable, and the third had a higher percent of β-galactosidase-positive cells than adult or neonatal melanocytes expressing wild type *p16* and *MC1R* (Table 2). We expect to finalize Task 1 within the next 6 months.

It is known than normal cells undergo replicative senescence *in vitro*. We expected melanocytes harboring mutant *p16* to evade senescence. To test this, we have been comparing the doubling time of these cultures at early, versus late passage (Task 3). Upon testing 5 different cultures, we found that their doubling time became markedly longer at passage 9 or 10 than at passage 3 or 4, indicating reduced proliferation rate (Table 3). The doubling times of melanocytes heterozygous for *p16* mutation fell within the range of their counterpart with wild type *p16.* We will continue to follow up the cultures to determine for how many passages and doublings they will undergo, if they will reach replicative senescence, and whether or not the percent of senescent cells will increase with passage in culture.

Cell Strain	Age	*P16* Genotype	*MC1R* Genotype	Doubling Time (d)
17609, P.3	18	V126D	D294H/+	3.2
17609, P.8				9.5
17494, P.4	20	V126D	WT	3.3
17494, P.9				9.7
85627, P.4	35	V126D	R160W/+	4.4
85627, P.10				6.6
19971, P. 4	24	5'UTR-34G>T	D294H/+	3.3
19971, P.9				10.5
19972, P.4	21	5'UTR-34G>T	R160W/+	7.3
19972, P.9				15.5
17490, P.4	28	V126D	WT	5.3
59854, P.7	23	WT	R151C/+	7.3
26310, P.5	28	WT	WT	7.0
1583, P.6		WT	WT	4.6
1744, P.9		WT	WT	4.4

Table 3. Comparison of the doubling time of melanocytes with different *p16* and *MC1R* genotypes, and passage number. In red are additional adult melanocytes, and in blue are neonatal melanocytes.

Given the role of p16 and MC1R in regulating melanocyte proliferation, we analyzed the cell cycle profiles of melanocytes expressing different *p16* and *MC1R* genotypes, with or without exposure to UV (Task 3). Eight different cultures were tested whereby melanocytes were either un-irradiated or irradiated with a dose of 90 mJ/cm^2 that we previously found to cause cell cycle arrest, significant induction of DNA photoproducts, and moderate apoptosis (≤25%) in melanocytes that are wild type for *p16* and *MC1R*. Data from 2 representative experiments are included in Table 4. Cell cycle analysis using BrdU incorporation was conducted at 0, 6, 24, 48, 72, or 96 h post UV, with fresh medium added daily. In the absence of UV exposures, all cultures, regardless of their genotype, showed a marked increase in the percent of cells in S phase, accompanied with a reduction in the percent of cells in G1 phase, and in most cases, an increase in the percent of cells in G2-M within 48 h of receiving fresh medium (Table 4). The corresponding UV-irradiated groups were cell cycle-arrested up to 72

Cell Strain	Treatment	G0-G1	S	G2-M
19972, P.4	Control 0 h	81.9	1.7	13.9
	Control 24 h	83.4	6.5	8.8
	Control 48 h	52.8	14.5	30.6
	Control 72 h	67.3	6.1	24.4
	Control 96 h	65.3	10.9	20.3
	UV 90 0 h	81.9	1.7	13.9
	UV 90 24 h	84.7	1.2	12.2
	UV 90 48 h	84.9	1.6	11.7
	UV 90 72 h	82.6	2.2	12.8
	UV 90 96 h	65.8	10.9	19.1
17609, P.7	Control 0 h	88.0	0.2	9.3
	Control 24 h	80.1	10.7	7.9
	Control 48 h	58.7	9.7	28.5
	Control 72 h	75.6	4.0	18.5
	Control 96 h	73.0	3.7	20.1
	UV 90 0 h	88.0	0.2	9.3
	UV 90 24 h	85.8	1.0	11.8
	UV 90 48 h	82.7	0.3	13.3
	UV 90 72 h	78.1	0.3	15.2
	UV 90 96 h	70.7	1.3	17.5

Table 4. Cell cycle analysis of melanocytes with different *p16* and *MC1R* genotypes under control conditions or after irradiation with 90 mJ/cm^2 UV.

hours, and began to recover within 72- 96 h post UV (Table 4).

Cell Strain	Treatment	G0-G1	S	G2-M
17489, P.4	Control 0 h	90.0	0.3	6.3
	Control 24 h	88.2	4.6	5.2
	Control 48 h	63.2	11.0	23.0
	Control 72 h	76.5	5.5	16.2
	UV 75 0 h	90.0	0.3	6.3
	UV 75 24 h	91.9	0.3	6.2
	UV 75 48 h	92.1	0.4	5.9
	UV 75 72 h	84.0	5.7	8.1
105, P.12	Control 0 h	71.7	0.8	21.2
	Control 24 h	67.1	9.1	16.9
	Control 48 h	46.5	8.6	34.5
	Control 72 h	49.1	10.7	30.8
	UV 75 0 h	71.7	0.8	21.2
	UV 75 24 h	71.8	1.2	21.8
	UV 75 48 h	70.7	1.6	21.3
	UV 75 72 h	58.9	8.7	24.4

Table 5. Cell cycle analysis of melanocytes with different *p16* and *MC1R* genotypes under control conditions or after irradiation with 75 mJ/cm^2 UV.

We had expected expression of *p16* mutations to allow melanocytes to bypass the UV-induced cell cycle arrest due to lack of inhibition of cyclin/cdk complexes, and co-expression of a *MC1R* loss-of-function variant to contribute further to this effect. We reasoned that the results we obtained that are presented in Table 4 could possibly be due to the relatively high dose of UV used. For that, we compared the responses of 3 different cultures to a lower dose of UV, namely 75 mJ/cm^2. Data of 2 representative experiments are included in Table 5. The growth arrest induced by this lower dose of UV was less prolonged than that induced by 90 mJ/cm^2, with significant increase in the number of S phase cells and reduction of G1 cells observed at 72 h post UV (Table 5).

To confirm the results presented in Table 5, we compared the doubling time of cultures by establishing growth curves under control, and UV-irradiated conditions using a dose of 75 mJ/cm^2. We compared the cell numbers at 3 (time of irradiation), 5, 7, 9, 11, 13, or 15 days post UV, with fresh medium added every other day. Ten different melanocyte cultures were tested, with passage number ranging between 2 and 9. Under control conditions, all cultures showed maximal increase in cell number on day 7 after plating. In response to UV, there was no increase in cell number at this time point, which corresponded to 4 days post UV exposure, and the subsequent increase was lower than that in the control groups. The initial UV-induced growth arrest, accompanied in most cases by reduced proliferation after partial recovery, resulted in a more prolonged doubling time of UV-irradiated melanocytes, which was observed in all 10 cultures, regardless of their genotype (Table 6). Despite the expected individual variation in the proliferation rate of melanocytes cultured from different donors, the population doubling times of melanocyte cultures heterozygous of a mutant *p16* allele, with or without expression of a mutant *MC1R* variant proliferated were comparable to those of adult melanocyte cultures expressing wild type *p16*, at similar passage number (Table 6). One exception is 84891 with the longest doubling time, which questions the differential impact of the 32ins24 mutation in *p16.* This will be confirmed in further experiments whereby two other cultures expressing the same mutation, 87203 and 87204, will be tested.

Cell Strain	Age	*P16* Genotype	*MC1R* Genotype	Control (d)	UV 75 (d)
19971, P.9	24	5'UTR-34G>T/+	D294H/+	10.5	13.0
84891, P.4	22	32ins24/+	R160W/+	13.4	18.3
27856, P.9	31	+/+	R151C/R151C	11.3	12.7
10766, P.5	42	V126D/+	R160W/R151C	7.0	8.2
17489, P.6	31	V126D/+	D294H/+	6.1	7.0
85627, 10	35	V126D/+	R160W/+	8.8	10.4
17490, P.2	28	V126D/+	WT	4.9	6.4
114, P.3		+/+	V92M/R151C/T314T	9.1	10.4
116, P.5		+/+	R160W/+	4.8	5.5
117, P.3	43	+/+	+/+	7.0	9.7

Table 6. Comparison of the doubling times of melanocyte cultures with different p16 and MC1R genotypes with or without irradiation with 75 mJ/cm^2 UV.

Almost all familial melanoma cases are heterozygous for *p16* mutations. We surmised that the reason melanocyte cultures heterozygous for one *p16* and/or *MC1R* mutant allele responded similarly to UV as wild type melanocytes was due to compensation by the functional allele of *p16* or *MC1R*. To investigate this possibility, we have available melanocytes

from patients homozygous or compound heterozygous for *MC1R* loss-of-function variants. Additionally, we recently had the privilege to obtain 2 different melanocyte cultures from the two rare individuals worldwide who are totally p16 null, from Dr. Dorothy Bennett, St. George's Hospital Medical School, London, U.K. These two individuals were homozygous for germline mutation that caused a deletion extending 16 bp and removing nucleotides at positions 218-237 of exon 2 of *p16* (Gruis, N et al., Nat Genet, 10:351-353, 1995). Having *p16* null melanocytes in hand will allow us to clearly delineate the impact of p16 loss on the response of melanocytes to UV and to understand the mechanism(s) by with *p16* acts as a melanoma predisposition gene.

Consistent with Dr. Bennett's previous findings (Sviderskaya E. et al., J Nat Cancer Inst 95:723-732, 2003), we found that these cultures have more stringent requirements for proliferation than melanocytes that are wild type or heterozygous for a mutation in *p16*. Using the growth medium we routinely use for culturing adult melanocytes, we found that one of the p16 null cultures failed to proliferate even after 3.5 months, and that the second had a very poor proliferation rate. Based on these results, we have been maintaining these cultures in an enriched medium consisting of our routine melanocyte growth medium supplemented with 10% fetal bovine serum (compared to 4% in the routine medium), twice the concentration of bovine pituitary extract, and 10 nM endothelin-1. This enriched medium resulted in marked proliferation of these cells, with 78% increase in cell number in the first 6 days, 68% increased between days 6-12, and 30% increase between days 12-18, as depicted in the growth curve of one of these *p16-/-* cultures, with a doubling time of 5.4 days, as shown in Fig. 4.

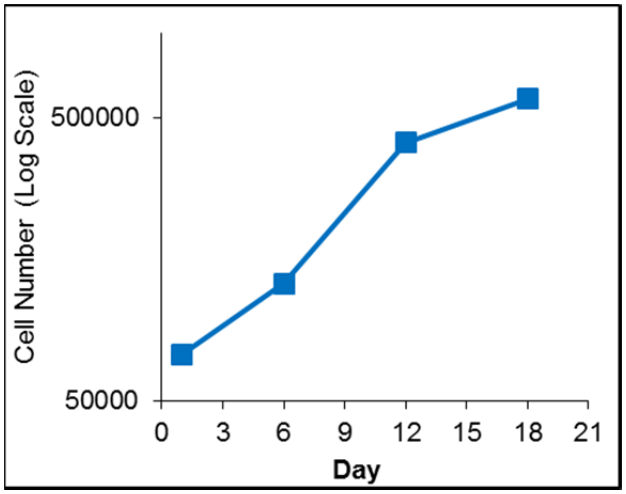

Figure 4. Growth curve of p16$^{-/-}$ melanocytes maintained in enriched melanocyte growth medium. Melanocytes were plated onto 6-well plates, at a density of 0.073x10^6 cells/well, and counted on days 6, 12, and 18. Each data point represents the mean cell number of triplicate wells. All standard errors were less than 10%.

Based on the above experiments testing the responses of melanocytes to a single irradiation with UV, we investigated the effect of repetitive UV irradiations with a low non-apoptotic dose of 25 mJ/cm^2, once every other day, on the proliferation of one of the *p16-/-* melanocytes maintained in our enriched medium (Fig. 5). A significant reduction in proliferation was observed after 4 irradiations, and this was comparable to the decrease resulting from a single irradiation of naïve melanocytes with 90 mJ/cm^2 UV. Repetitive exposures to low doses of UV is thought to confirm photoadaptation, which should reduce the damaging effect of a subsequent high dose of UV. To test this, we irradiated the p16-/- melanocytes 3 times with 25 mJ/cm^2, and 48 h later, with 90 mJ/cm^2. This regimen further inhibited proliferation beyond that observed when these melanocytes were only irradiated once with 90 mJ/cm^2 UV, suggesting lack of photoadaptation. This preliminary experiment will be repeated using p16-/- melanocytes and melanocytes with different *p16* and *MC1R* genotypes, to determine the effects of multiple irraditions on proliferation, as well as induction and repair of DNA photoproducts and apoptosis.

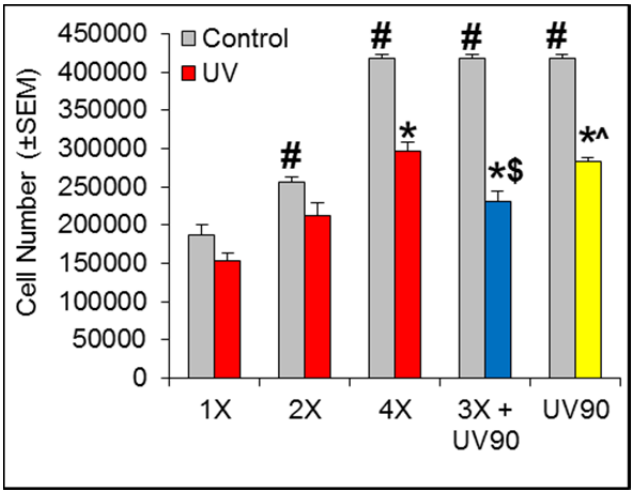

Figure 5. Effect of multiple irradiations with a low dose of UV on the proliferation of p16$^{-/-}$ cells. Melanocytes were plated at a density of 0.15x10^6 cells/well in 6-well plates. On day 3, and every other day thereafter, melanocytes were either unirradiated, or irradiated with 25 mJ/cm^2 UV, and counted 48 h after each irradiation (1x, 2x, and 4x). Two additional groups were included whereby melanocytes were irradiated 3x with 25 mJ/cm^2 UV followed 48 h later by irradiation with a high dose of 90 mJ/cm^2 UV, or irradiated only once with 90 mJ/cm^2 UV. Each data point represents the mean cell number of triplicate wells/group ±S.E. #= Statistically different from the earlier control at p<0.001; *= statistically different from the its respective control at p<0.001; $= statistically different from 4x UV group at p<0.01; ^=statistically different from 3xUV+UV90 at p<0.05, as determined by Anova.

Conclusions: The data we have obtained so far, relevant to Tasks 1 and 3 of Specific Aim II, do not reveal any striking differences between the proliferation of melanocyte cultures heterozygous for *p16* mutation, with or without expression

of a loss-of-function *MC1R* variant, and melanocytes wild type for *p16* and either wild type for *MC1R* or heterozygous for a loss-of-function *MC1R* variant. Also, expression of one mutant *p16* allele with or without a *MC1R* mutant allele did not enable melanocytes to escape UV-induced growth arrest and did not seem to allow them to evade senescence. One possible exception is the 32ins24 mutation in *p16,* which might have a greater impact than V126D or 5'UTR-34G>T mutations on melanocyte proliferation.

Results relevant to Specific Aim III.

The proposed experiments were initially planned to begin during the second year of funding. However, during the past summer, we began investigating the effects of UV on activation of NFκB (Task 1). For that, we have compared the protein levels of phospho-IKKα/β, phospho-IκB, and phospho- NFκB, vs. the respective total proteins, in control, UV-irradiated, or hydrogen peroxide-treated melanocytes, using Western blot analysis (Fig. 6). As a positive control, we included lysates of cells treated with TNF-α, known to activate the NFκB pathway. We have so far tested 3 different cultures expressing wild type *p16*, and 17609. We found that treatment with hydrogen peroxide increased the phosphorylation of IKKα/β within 15 minutes, and this effect declined, but remained above control 6 h after treatment. Phosphorylation of NFκB was first evident at 15 min, but persisted for 6 h after hydrogen peroxide treatment. Irradiation with UV at either 90 or 105 mJ/cm^2 had no effect on either IKKα/β or NFκB, at any of the time points tested, which ranged from 15 min to 28 h. Neither hydrogen peroxide, nor UV had any effect on IκB, the substrate of IKKα/β, which binds to, and inhibits NFκB. The two doses of UV we used induce transient, yet substantial reactive oxygen species, based on our previous quantification of UV-induced hydrogen peroxide generation, and activate antioxidant enzymes, such as catalase. We are still exploring why UV failed to activate the NFκB pathway in melanocytes, and whether this effect of UV is melanocyte-specific.

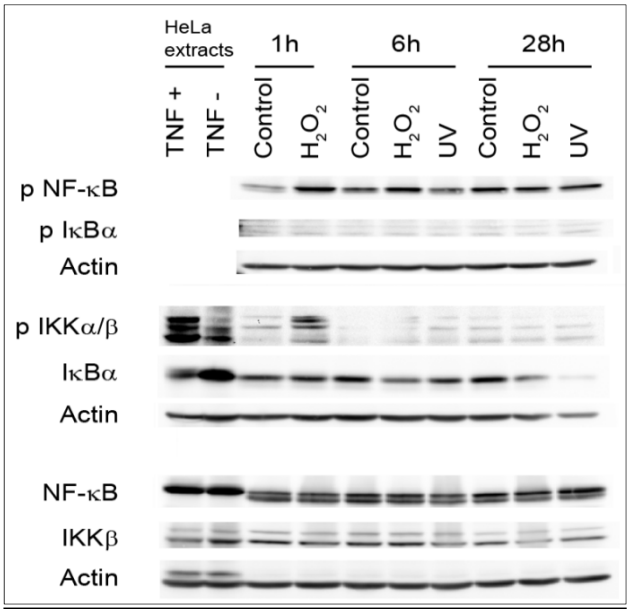

Figure 6. Activation of IKKα/β and NFκB by hydrogen peroxide, but not UV in melanocytes. Western blot analysis was carried out on total cell lysates derived from melanocytes irradiated with a dose of 90 mJ/cm^2 UV, or treated for 30 min with 250 μM hydrogen peroxide, at 1, 6, and 28 h post UV exposure or 30 min treatment with hydrogen peroxide. Untreated controls were included for each time point, and lysates of HeLa cells, either untreated, or treated with TNF-α were used as a positive control. The levels of phospho, and total IKKα/β, IκB, and NFκB were detected, and actin was used as a loading control.

Since it has been reported that loss of *p16* and loss of function of MC1R increase oxidative stress in melanocytes, and the latter results in sustained generation of hydrogen peroxide following UV exposure, we compared the production of hydrogen peroxide in melanocytes with different *p16* and *MC1R* genotypes in response to irradiation with 90 mJ/cm^2 UV (Table 7). Upon testing 9 different cultures with different *p16* and *MC1R* genotypes, we found that co-expression of a mutant *p16* and *MC1R* alleles did not contribute to basal or UV-induced hydrogen peroxide levels, as

Cell Strain	*P16* Genotype	*MC1R* Genotype	Basal H_2O_2 (pmol/ml/10^6 cells)	UV-induced H_2O_2 (pmol/ml/10^6 cells)	% increase
105	+/+	+/+	24.0±1.1	57.5±4.8	239
114	+/+	V92M/R151C/ T314T	56.4±9.3	195.3±5.9	346
116	+/+	R160W/+	57.7±8.8	147.3±7.1	255
17490	V126D/+	+/+	57.6±8.4	180.14.4	313
17494	V126D/+	+/+	40.4±3.1	103.1±11.7	255
17489	V126D/+	D294H/+	34.7±6.7	86.1±6.4	248
17609	V126D/+	D294H/+	63.9±0.5	125.8±9.7	197
85627	V126D/+	R160W/+	47.8±2.9	112.6±19.9	236
19971	5'UTR-34G>T/+	D294H/+	46.3±8.5	229.6±15.7	496

Table 7. Comparison of basal and UV-induced H_2O_2 levels of melanocytes with different *p16* and *MC1R* genotypes.

compared to expression of either *p16* or *MC1R* mutant allele, or wild type *p16* and/or *MC1R* (Table 7).

Conclusions: UV failed to activate the NFκB pathway in melanocytes regardless of their *p16* or *MC1R* genotype. However treatment with hydrogen peroxide, which is expected to cause oxidative stress, induced the phosphorylation, hence the activation of IKKα/β and NFκB. Co-expression of a mutant *p16* and *MC1R* allele does not seem to augment the basal level of hydrogen peroxide or increase its production upon UV exposure.

What opportunities for training and professional development has the project provided?

This project provided a training opportunity for Steve Guard, who was a previous undergraduate SURF-ASPET student in my laboratory in summer of 2014. He returned to my laboratory in summer of 2015 and assisted in obtained some of the data included above, particularly the results of growth curves, β-galactosidase staining, and Western blotting for IKKα/β, IκB, and NFκB. He is currently a graduate student in the Cancer Biology Program at University of Colorado, Boulder.

How were the results disseminated to communities of interest?

Nothing to report

What do we plan to do during the next reporting period to accomplish the goals?

We plan to conduct further experiments comparing melanocyte cultures heterozygous for *p16* and/or *MC1R* mutations to melanocytes that are *p16-/-*, and accomplished all the remaining tasks of Specific Aims I, II, and III. The results to be obtained will be included in at least one manuscript to be submitted for publication within the next 6 months. We also plan to submit our results as abstracts to be presented at the meeting of Society for Investigative Dermatology and the Pan American Society for Pigment Cell Research. We are planning to recruit patients from OHSU in order to obtain skin biopsies to increase the n number of our cultures and hence the statistical power of our results.

4.Impact

What was the impact of the development of the principal discipline(s) of the project?

To our knowledge, this is the first attempt to methodically address the impact of 3 mutations in *p16* expressed in familial melanoma cases, with or without co-expression of a non-functional *MC1R* allele on melanocytes. It is critical to test melanocytes, since mutations in *p16* increase the risk for melanoma, but not other skin cancers, such as basal or squamous cell carcinomas that are derived from keratinocytes. More than 99% of patients from melanoma-prone families are heterozygous for *p16* mutations, hence the melanocyte cultures we have established are true representative of these high-risk patients. Previous reports on *p16* mutations relied on silencing the gene in melanocytes and other cell types, such as fibroblasts, or in mouse models. Our consistent findings that melanocytes heterozygous for a mutant *p16*, even in the presence of a *MC1R* mutation, do not have an obvious aberrant response to acute UV exposure suggest that further stressors or other "hits" are needed for melanocyte transformation to melanoma. Based on our findings, we hypothesize that these melanocytes might to be more sensitive than normal melanocytes to the challenge with chronic UV exposure, which might overwhelm their DNA damage response and other compensatory mechanisms. It is also conceivable that germline *p16* mutations alter the microenvironment of melanocytes in the skin, hence keratinocytes and fibroblasts might contribute to the mutator phenotype of melanocytes. Accomplishing the aims of this project will lead to novel findings that should improve our understanding of the underlying mechanisms for melanoma in high risk individuals, particularly members of melanoma-prone families harboring germline mutations in *p16* and *MC1R*.

What was the impact on other disciplines?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

We anticipate that the outcome of this project will provide accurate biomarkers (*p16* and *MC1R* genotype) that will allow for more precise assessment of melanoma risk, and more thorough and stringent follow up of high risk individuals for melanoma prevention and early detection.

5. Changes/Problems:

Nothing to report.

6. Products:

Nothing to report

7. Participants and Other Collaborating Organizations:

What individuals have worked on the project?

Name:	Zalfa Abdel-Malek
Project Role:	PI
Nearest person month worked:	2.4
Contribution to project:	Maintained cell cultures and performed experiments; participated in all experimental designs and data analysis; was in charge of communicating with collaborators, and supervising her laboratory staff.
Name:	Viki Swope
Project Role:	Senior Research Associate
Nearest person month worked:	4.2
Contribution to project:	Assisted in maintenance of cultured cells; performed cell cycle analysis (Specific Aim II, Task 3, and assisted in Western blot experiments pertinent to Specific Aim III, Task 1; assisted the PI in data analysis and interpretationβ
Name:	Renny Starner
Project Role:	Senior Research Assistant
Nearest person month worked:	4.2
Contribution to project:	Helped in maintaining cultured melanocytes; performed DNA repair and apoptosis experiments (Specific Aim I, Task 2); assisted in growth curve experiments (Specific Aim II, Task 3) and in data analysis
Name:	Ana Luisa Kadekaro
Project Role:	co-Investigator
Nearest person month worked:	0.6
Contribution to project:	Assisted in the Western blots and measured hydrogen peroxide levels in various cultures (relevant to Specific Aim 1 Task I and Specific Aim III, Task 1); assisted in data interpretation
Name:	Steve Guard
Project Role:	Temporary Research Assistant (summer 2015)
Nearest person month worked:	2
Contribution to project:	Assisted in cell cycle analysis, growth curves, β-galactosidase staining (Specific Aim II, Tasks 1 and 3), and western blot analysis for the NFκB pathway (Specific Aim III, Task 1).

Funding support: Departmental funds

Change in active other support of the PD/PI or senior/key personnel:

Type of award: R21 CA191761

Title: *Melanoma Prevention by MC1R Small Peptide Analogs of α-MSH*

Funding agency: National Cancer Institute

Funding period: 7/1/15-6/30/17

Total cost: $406,258

What other organization was involved as partner?

Organization name:	Oregon Health and Sciences University
Location of Organization:	Portland, Oregon
Partner's contribution to the project:	Drs. Leachman and Cassidy have been instrumental in lending their extensive knowledge about p16 and melanoma predisposition, and valuable advice on experimental design (chronic versus acute UV exposure; dosage of UV to use), and assisted in data interpretation. Dr. Cassidy has developed assays to measure the oxidative state of melanocytes, namely measurement of glutathione levels, which will be applied to the mutant cultures used in this project. Upon obtaining the final approval from CDMPR regarding human subjects, they will be actively recruiting patients to include in this project.

8. Special Reporting Requirements:

Nothing to report

9. Appendices:

Nothing to report